5-2011

The Antimicrobial Activity of Cold Pressed Terpeneless Valencia Orange Oil at Cold Temperatures

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

By

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Bachelor of Science in Chemistry, 2008

May 2011
University of Arkansas

Abstract

This research was undertaken to identify an antimicrobial for use during chilling in beef processing, including periods of temperature abuse, as well as to evaluate membrane filtration as means to remove essential oils from bacteria to prevent further antibacterial action. Cold pressed terpeneless Valencia orange oil was examined in combination with various temperatures (37°C, 10°C, and 4°C) to determine its antimicrobial activity against various strains of bacteria. The strains were tested using a ninety-six well microtiter plate method, with or without membrane filtration. The compound 2, 3, 5-triphenyl tetrazolium chloride was used as a growth indicator. Serial, two fold dilutions of the oil were tested. Plates were incubated at 37, 10, or 4°C for times up to 48 hours. Under the non-filtration method, 6 hours of exposure to the oil at 37°C, resulted in strains of *E. coli* O157:H7 being inhibited at oil concentrations ranging from 0.6% to 0.2%, with a mean of 0.4±0.01%. At 10°C, the O157:H7 strains were shown to be inhibited at concentrations ranging from 1.4 to 0.9%, with a mean of 1.1 ± 0.2%, after 6 hours. At 4°C, O157:H7 strains were inhibited after 6 hours at concentrations ranging from 4.6 to 2.3%, with a mean of 3.5 ± 2.1%. Under the membrane filtration method, *E. coli* O157:H7 was inhibited by cold pressed terpeneless Valencia orange oil at a concentration of 0.5±0.0%, *L. monocytogenes* at 0.5±0.0%, *S. aureus* at 0.31±0.13%, *S.*Typhimurium at 0.31±0.13%, *S. sonnei* at 0.75±0.29%, *Y. enterocolitica* at 0.31±0.13%, *E. faecalis* at 0.63±0.25%, *B. cereus* at 0.44±0.13%, and *P. aeruginosa* exhibited complete resistance to the oil. The ranges of MICs found under the non-filtration method appear to be the result of effects from the variable nature of a complex media and an antimicrobial that presents potential multiple mechanisms for inhibition of *E. coli* O157:H7 at refrigeration temperatures. The MICs found under the membrane filtration method were not significantly greater than those found by prior studies,

indicating that membrane filtration is not required for determination of minimum inhibitory concentrations for essential oils.

This thesis is approved for
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Acknowledgements

I want to extend my most sincere gratitude to my major professor, Dr. Philip Crandall, and the members of my committee, Dr. Steven Ricke and Dr. Joshua Sakon. Their support and guidance was essential for my Master's career. I am also very grateful to Dr. Larry Goodridge for his help and input on my work. His insights were a great help in developing and completing my research. I would like to thank Dr. Betty Martin for her help in teaching me the ropes of microbiology. Her continued support during my time in the Food Science Department has been inspirational. I would also like to thank the National Cattleman's Beef Association for the funding that made my research possible.

I would also like to acknowledge those whose lab assistance contributed greatly to this research. The time and effort put forth in the lab by Ashley Clement, Erin Shannon, and Sujata Sirsat was invaluable. I also want to acknowledge Robert Story for his assistance in the lab and always being there for my questions. I truly appreciate the help he has given me during my Master's career.

Lastly, I would also like to thank Dr. Irene Hanning for her mentorship, her help in the lab, and for supplying me with new and interesting opportunities inside and outside of the lab. Her encouragement and support helped me greatly to complete my degree.

Table of Contents

Introduction

There are an estimated over 170,000 cases of shiga toxin producing *E. coli*, (STEC) in the United States each year. Of these cases, approximately 2,400 result in hospitalization. A major source of STEC infections is ground beef, due to the colonization of *E. coli* O157:H7 in the gut of many cattle and subsequent spread of this pathogen to other parts of the meat during processing. Processing of cattle includes removal of the hide, evisceration, trimming to remove visible contamination, carcass washing, and ultimately chilling. Most intervention steps occur prior to chilling and therefore any O157:H7 contamination present at the chilling stage has potential to be passed on to the consumer. The fact that O157:H7 outbreaks are still occurring indicates additional hurdles during or post chilling are required to prevent O157:H7 from entering the retail product stream.

In the late 19th century, the antimicrobial properties of essential oils were first explored. Although essential oils are mainly used as flavor and fragrance, there has been a recent emphasis on the possible role of essential oils as antimicrobials due to an increase in consumer demand for all natural and organic products. Essential oils vary in effectiveness against different strains of bacteria. Given this variability, there is a need for more research into essential oils and their antimicrobial applications under specific environmental conditions to optimize their application in food systems.

The chilling stage of beef processing seeks to rapidly bring beef carcasses to 4°C. Once this temperature is reached, retailers attempt to maintain this temperature as a part of a "cold chain" until the consumer stores the hamburger in their home refrigerator. Application of an essential oil antimicrobial at this stage of processing could give processors an additional intervention at the end of processing, resulting in a lower chance to pass potential pathogens,

like *E. coli* O157:H7, on to the customers. Thus reducing the number of outbreaks and recalls, as well as increasing consumer confidence in beef products.

The main methods for determining minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of essential oils and essential oil components are broth dilution, agar diffusion, and disc diffusion. These methods do not employ a neutralization step, indicating that the compounds being tested are still acting on the bacteria when MICs and MBCs are observed. In order to eliminate this effect one possible method is membrane filtration. This involves filtering volumes of treated cells through a membrane, in order to separate out the cells from antimicrobial treatments. The filters can be washed, then placed on agar, and incubated for 24 hours. Plates can then counted to determine viable cells. Membrane filtration offers the ability to remove antibacterial compounds without the need for neutralizers, especially when an adequate neutralizer is not available, as is the case with complex essential oils. This aspect of membrane filtration indicates its promise for the use in essential oil MICs and MBCs.

The current research was undertaken to increase understanding of the antimicrobial properties of cold pressed terpeneless Valencia orange oil against *E. coli* O157:H7, to determine the efficacy of the oil as an intervention at the chill stage of bee processing, and to examine the efficacy of membrane filtration in the determination of minimum inhibitory concentrations.

Literature Review

Abstract

Since the major, multistate outbreak of *Escherichia coli* O157:H7 in hamburgers in 1992-1993, in which 501 people became ill and three ultimately died of their infection, *E. coli* O157:H7 and other hemorrhagic *E. coli* (EHEC) have been a major concern for the beef industry. The beef industry has developed hazard analysis and critical control point (HACCP) plans to reduce or eliminate these pathogens, but even with these plans in place there are still outbreaks of this pathogen associated with beef. As recently as the Fall of 2009, there was a multistate outbreak in which 248,000 pounds of beef products had to be recalled due to possible contamination with *E. coli* O157:H7. The most common technologies used to decontaminate beef carcasses range from simple cutting away of visually contaminated portions of the carcass, to carcass steaming, or application of antimicrobials using spray cabinets. With the rise of consumer preference towards all natural and organic products, beef processors may need to look for alternatives to the current chemical antimicrobials. This review investigates the current technologies employed in the beef industry to reduce pathogens during processing and the current knowledge of how essential oils and their components act as antimicrobials in order to determine their potential as an all natural solution to pathogen reduction during beef processing.

Introduction

Since 1982, *Escherichia coli* O157:H7 has been a major concern in the food industry, and specifically in the cattle industry, due to its pathogenic nature (Wells et al. 1983). Cattle have been identified as a main reservoir of O157:H7 (Chapman et al. 1993). Additionally, serological evidence suggests that all herds of cattle have some exposure to this pathogen (Laegreid et al. 1999). The transfer of this organism from cattle to man can lead to serious illness, hospitalization, and even death. It is known to cause hemorrhagic colitis, which involves abdominal cramping and bloody diarrhea (CDC 1983), and extreme cases can lead to hemolytic uremic syndrome (HUS). HUS is characterized by the destruction of red blood cells, kidney injury, and potentially death. The cattle industry has sought to eliminate this pathogen and others via various means.

Non-chemical interventions

Trimming

Physical measures are used in the cattle industry to reduce microbial loads, and reduce the prevalence of *E. coli* O157:H7. One of these is trimming of areas of gross fecal contamination on the carcasses. *E. coli* O157:H7 is an intestinal pathogen and therefore is transmitted to the meat via intestinal contents and fecal matter. When cattle enter an abattoir, their hides are commonly covered in fecal matter which could potentially contain *E. coli* O157:H7. During slaughter, hides are removed, and thus much of the fecal matter is removed from the outside of the carcass, but occasionally some is transferred to the carcass. This leads to the need for spot trimming. Trimming works by simply removing areas that appear visually to be contaminated with fecal matter. Trimming has been shown to reduce *Salmonella* Typhimurium and *E. coli* O157:H7 by approximately 3 logs (Hardin et al. 1995, Castillo et al. 1998b, and Phebus et al. 1997).

Water Washes

The use of warm or hot water washes to remove bacteria from carcasses has been proven to be an effective form of intervention. Water interventions using temperatures near 35°C are considered warm water washes, while interventions using temperatures from 70°C and above are considered hot water washes (Dorsa et al. 1997 and Phebus et al. 1997). Water washes work via physical removal of the pathogen from the surface and, in the case of hot water washes, the thermal inactivation of the bacteria. In 1993, Barkate et al. examined the effect of hot water on the reduction of bacteria on beef carcasses. They found that a 95°C hot water wash was able to reduce bacteria by approximately 1 log. In 1996, Dorsa et al. examined the effects of hot water, as well as warm water, on the decontamination of beef and sheep carcasses. It was determined that for the removal of *E. coli* from beef, warm water (30°C) applied at 125 psi was just as effective as hot water (72°C) applied at 20 psi. Both treatments were found to reduce *E. coli* contamination by approximately 2.6 logs. In 2000, Cutter et al. replicated this result with a slightly higher temperature (35°C). They determined that *E. coli* O157:H7 was reduced by 2.5 logs when treated with 35°C water at 125 psi. Two studies by Castillo et al. (1998a,b) found that warm water at 35°C was able to reduce *E. coli* O157:H7 and *Salmonella* Typhimurium by approximately 2 logs when applied at a pressure starting at 250 psi and increasing to 400 psi during treatment. Dorsa et al. (1997a) demonstrated that hot water at 70°C and 40 psi produced a 2.6 log reduction in *E. coli* O157:H7, the same reduction seen at 2 degrees higher and 20 psi lower in their prior study. This result could possibly indicate that the temperature, and not the pressure, is the major factor in the reduction, although this doubling of the pressure does not even approach the pressures used for warm water in the prior study. To completely rule out the effect of pressure on bacterial decontamination, one would need a test of both warm water and hot water at the same pressure. Testing of the effect of water

pressure and temperature on decontamination carried out by Gorman et al. (1995) determined that higher pressures did increase bacterial reductions slightly (less than 1 log), but ultimately the temperature was found to be the most important factor. Beef surfaces treated with 74°C water at 300 psi followed by water at 16°C and 20 psi were found to have received a three log reduction from the untreated control. Beef surfaces treated with water at 16 or 35°C at 300 psi followed by water at 16°C and 20 psi only received approximately a one log reduction. The results presented by Gorman et al. (1995) indicate that at high pressures, temperature is the major factor in play for bacterial reductions. Cutter et al. (1997) investigated the parameters that affect spray washes on beef. Their investigation into temperature revealed that there was no significant difference in the reduction in their target bacteria, *E. coli* O157:H7, by water sprays at 30, 40, 50, 60, or 70°C. While Cutter et al. (1997) used a lower pressure to administer their sprays (80 psi), this is most likely not the reason for the difference in results. Cutter et al. (1997) used lean carcass tissue to perform their experiments, while Gorman et al. (1995) used adipose tissue. It has been found that antimicrobial effects can be greater on adipose tissue when compared to lean tissue (Dickson 1991, Cutter and Siragusa 1994, and Marshall et al. 2005). Although, Cutter et al. (1997) did not find any significant difference between reduction of *E. coli* O157:H7 when sprayed with different temperatures, they did find that all water sprays reduced the pathogen by approximately 2 logs.

Steam pasteurization

Steam can be used to convey great amounts of thermal energy onto a surface, and thus pasteurize it. The use of steam to pasteurize food was explored early in the 1970's by Klose and Bayne (1970) and Klose et al. (1971) in order to control *Salmonella* on chicken. Klose and Bayne (1970) found that chicken drum sticks exposed to water at 76°C under reduced pressure, to produce steam at the low temperature, for up to 8 minutes reduced *S.* Typhimurium by 5 logs.

In a subsequent experiment Klose et al. (1971) used steam pasteurization to decontaminate whole birds and bird parts. When the whole birds were exposed to steam at 71-73°C for 4 minutes, only a 1 to 2 log reduction was observed, but bird halves showed a 3 log reduction and drumsticks showed a 4 log reduction. Their conclusion as to the difference was that in order to get better reductions the steam needs to penetrate into the cavities of the bird Biemuller et al. (1973) also looked at the use of steam to pasteurize the surface of pork carcasses. They applied the steam onto inoculated areas from approximately 1 inch from the surface for 10 seconds. The steam treatment was able to reduce *Salmonella* Enteritidis by more than 5 logs. While this is a positive result, they found that the steam treatment caused the skin to shrink and turn slightly yellow, as well as causing a darkening of the blood vessels. Anderson et al. (1979) used steam to control microbial growth on plate beef. They used 95°C steam to pasteurize the beef surface, but found that it was ineffective. The aerobic plate counts were only reduced by less than 1 log, and subsequently increased when stored for up to 12 days. These early short comings lead to a lull in the research of steam pasteurization until the mid 1990s, at which time research began to pick up. Dorsa et al. (1996) published a paper showing that combinations of water washing, air-drying, and steam treatments followed by a final water wash were able to produce 1 to 4 log reductions of aerobic plate counts (APCs) on sheep carcasses depending on water temperature used to wash the carcass initially and the initial inoculum level. The greatest reduction was seen when 82.2°C water was used to wash the carcass initially and the inoculum level was set at approximately 7 logs, although the greatest reduction seen was not significantly different from a treatment of only the 82.2°C water wash. At lower initial water temperatures (54.4 and 15.6°C) the steam pasteurization treatment proved to be significantly better than only water washes. At 15.6°C and an approximately 7 log inoculum, the single water washes reduced APCs by approximately 2 logs, while the steam pasteurization treatment reduced APCs by 3 logs. Phebus

et al. (1997) looked at the use of a novel steam pasteurization chamber for effectiveness at reducing bacterial pathogens on beef carcasses. Meat samples were exposed to pressurized steam for 15 seconds and then cooled with 1°C water for 20 seconds. They found that *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were all reduced by approximately 3.5 logs. Later, Nutsch et al. (1997) took the novel steam pasteurization chamber to the commercial level. They found that APCs were reduced by approximately 1 log after treatment and chilling, down from 2 logs initially. They also found that generic *E. coli* populations were undetectable after treatment and chilling, although initial levels were only 0.6 to 1.2 logs and the detection limit was 0.6 logs, generic *E. coli* was found on 10 of 70 carcasses before treatment and on none of the carcasses after treatment. In a similar study by Gill and Bryant (1997), it was determined that steam pasteurization was able to reduce generic *E. coli* counts from 3.84 logs to undetectable levels after treatment and chilling. Nutsch et al. (1998) looked at the differences steam pasteurization had on different anatomical sites. They found that *E. coli* was reduced by approximately 0.5 logs in all locations from an average initial population of 1 log. Overall, all bacteria tested were significantly reduced by the steam pasteurization process, though none saw reductions greater than 1.5 logs, the greatest initial population of any bacteria, other than APCs, was 2.5 logs. APCs were initially in the 3 to 4 log range. Retzlaff et al. (2004) studied a vertical tower static chamber steam pasteurization unit to determine its ability to reduce bacterial populations of *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria innocua* on beef carcasses. They found that steam at 98.9°C was able to reduce pathogen populations by 4.6-4.8 logs when the samples were exposed to the treatment for 15 seconds. Advances in steam pasteurization technology have allowed this intervention method to go from an ineffective and damaging process to a viable option in the beef industry.

Chemical interventions

Cetylpyridinium chloride

Cetylpyridinium chloride (CPC) is a compound commonly found in dental hygiene products (Beers et al. 2006). Cutter et al. (2000) proposed the use of CPC to reduce pathogenic bacteria on beef by use as a prechill spray. Prior to this time, its potential use as a carcass intervention was mainly seen in the poultry and catfish industries (Kim and Slavik 1996, Xiong et al. 1998, Yang et al. 1998). Cutter et al. (2000) found 1% CPC to be extremely bactericidal against *E. coli* O157:H7 and *Salmonella* Typhimurium, finding approximately 6 and 5 log reductions, respectively, immediately after treatment, as compared to an untreated control. Although these results are very positive, the residual CPC found on the carcasses was found to be in excess of the daily allowed intake of CPC, indicating that at 1% CPC is a wonderful antimicrobial, but leaves the product unsuitable for human consumption. The potential advantages of CPC, have led to further research. Bosilevac et al. (2004) looked at the use of CPC at the hide removal stage, and found that at this stage of processing the use of CPC led to cleaner carcasses down the line and ultimately a reduction in *E. coli* O157. Their study of residual CPC, found that by the end of the process the CPC levels were acceptable. Ozdemir et al. (2006) looked at the effect of CPC on *Listeria monocytogenes* and *Staphylococcus aureus* when applied to beef muscle. They found both bacteria were reduced by approximately 2 logs immediately following exposure to 0.5% CPC and after storage for up to five days. The residual CPC totals were not taken, so it difficult to say if the decreased concentration of CPC, as compared to Cutter et al.(2000) experimental parameters led to a safe level of CPC after treatment. As of May 2004, the FDA has approved the use of CPC for the decontamination of poultry carcasses (Beers et al. 2006), but as of March 2008, it has still not been approved for use on beef carcasses (Best Practices for Beef Slaughter).

Organic Acids

The most common method employed in the beef processing industry to reduce EHEC is an organic acid wash of the entire carcass after removing of the hide and evisceration and prior to chilling. The two organic acids of choice are currently lactic acid and acetic acid. Lactic acid works by lowering the environmental pH and, in at least the case of gram-negative bacteria, making the membranes more permeable (Alakomi et al. 2000). It can be assumed that most acids work in a similar mechanism. Organic acids have been shown to reduce bacterial loads on beef surfaces (Stopforth et al. 2004, Bacon et al. 2000, Castillo et al. 1998b, Dorsa et al. 1997b) and extend shelf life (Dixon et al. 1991, Smulders and Woolthuis 1985, Siragusa and Dickson 1992, Castillo et al. 2001a).These organic acids are generally delivered via a spray washer, at a concentration between 1 and 5% (Prasai et al. 1991, Cutter and Siragusa 1994, Dorsa et al. 1998a, and Dorsa et al. 1998b) at various temperatures (Brackett et al. 1994, Kang et al. 2001, Castillo et al. 2001a). Some researchers have shown that acetic acid is less effective than lactic acid (Anderson et al. 1992, Brackett et al. 1994, Dorsa et al. 1998a, Dorsa et al. 1998b, and Hardin et al. 1995), yet others have shown that there is not a significant difference between them (Acuff et al. 1987, Cutter and Siragusa 1994, Dorsa et al. 1996, and Siragusa and Dickson 1992). There are many conflicting reports in the literature. Some suggest that hot organic acids exhibit increased inhibition of bacteria (Van Netten et al. 1994, Anderson and Marshall 1989 and Anderson and Marshall 1990), while others indicate that they are ineffective (Brackett et al. 1994 and Conner et al. 1997) or do not increase antibacterial activity (Dickson and Anderson 1991). Overall, studies support the fact that organic acids reduce bacterial loads on beef and extend shelf life. Therefore they have been used as an antibacterial intervention in the beef industry.

Castillo et al. (2001a,b), suggest additional points of intervention for the use of organic acids. They examined the use of a 30 second post-chill spray, using lactic acid heated to 55°C at a 4% concentration. Using this procedure under experimental conditions, they found that the intervention gave an additional 2 log reduction of *E. coli* O157:H7 and a 1.6 log reduction of *Salmonella* Typhimurium. According to the Best Practices for Beef Slaughter (2008), the use of lactic acid at 55°C and at a concentration of up to 5% has been approved by FSIS. This gives beef processors another point of intervention in which they can reduce bacteria and bacterial pathogens.

While not in use in the beef industry, another intervention point for use of organic acids is during spray chilling. Spray chilling is normally performed with water or water mixed with chlorine, but Stopforth et al. (2004) suggest the addition of antibacterial compounds, such as an organic acid or another chemical into this spray, in order to give a second antibacterial intervention during processing. Prior work into this has not yielded very positive results. Heitter (1975) suggested the use of the Chlor-chil system, a spray chill system with added chlorine, in order to reduce carcass shrinkage and bacteria. While the system did reduce shrinkage, the reduction in bacteria was not significant. A similar result was found by Stevenson et al. (1978) when they examined the effects of chlorine spray on the beef. Hamby et al. (1987) examined the use of 1% lactic or acetic acid in a spray chilling system, and determined that while they did see reductions using the organic acids, that optimally they are best used right after slaughter. Dickson (1991) published a paper indicating that incorporation of 2% acetic acid into the spray chill treatment could lead to reductions of up to three logs when applied to beef adipose tissue, and reductions of up to one log on lean tissue for *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes.* Spray treatments involving only acid washes produced the highest reductions among all bacteria. Dickson and Anderson (1991) studied spray chilling with the

addition of 2% acetic acid, and found that populations of *Salmonella* California from lean beef tissues that grew on selective agar were reduced by the 2% acetic acid spray treatment, but that there was no difference in the counts on non-selective agar, indicating that cells were injured by the treatment, but were able to recover given the chance. On beef adipose tissue, it was determined that the addition of acid into the spray cycle only reduced *Salmonella* populations by less than 0.5 logs, and only when acetic acid was not used to sanitize the tissue prior to spray chilling. The result of this study indicate that adding acetic acid to the spray chill process did not greatly enhance pathogen reduction. Most published studies of the use of organic acids or other antimicrobials added to a spray chill method have not had positive results. A lack of current research in this area has left a large hole in our knowledge about additional points of intervention. In a time where multiple interventions are becoming standard, additional points of intervention can only help to reduce bacteria on carcasses. Further research is needed to evaluate the efficacy of these treatments in commercial abattoirs. Also, further research into the application of natural antimicrobials, such as essential oils and their components, in the spray chilling process is needed.

Essential Oils

The antimicrobial properties of essential oils are a recent focus in the world of food safety because of a desire on the part of many consumers to reduce the use of "hazardous chemicals" in their food (Williams et al. 2004, Williams and Hammitt 2001, and Schifferstein and Ophuis 1998). While there are many studies on the antimicrobial activities of essential oils, few take the next step and determine the mode of action of these compounds. In order to better use the compounds, we need to understand how they function.

Mode of action

Nearly every study agrees that the main mode of action for essential oils is by targeting the bacterial cytoplasmic membrane. Although the language varies, these studies indicate that these oils damage the cell membrane in such a way that leads to the death of the cell or, at the very least, prevent normal growth of the cell. In order to elicit a more precise mode of action, studies have looked at increases in membrane permeability, decreases in membrane potential, changes in ATP production, elimination of the pH gradient, and decreases in the respiration of the cell.

Increased permeability of the membrane

The leakage of potassium into the extracellular space is considered an indicator for increases in membrane permeability and ultimately loss of viability for the cell. This particular result was examined by multiple studies (Ultee et al. 1999, Cox et al. 2000, Cox et al. 2001, Lambert et al. 2001, Walsh et al. 2003, Fitzgerald et al. 2004, Inoue et al. 2004, Togashi et al. 2008, and Bouhdid et al. 2010). Ultee et al. (1999) examined carvacrol for its antimicrobial properties against *Bacillus cereus* and found that, at a concentration of 1mM, it caused an immediate decrease in intracellular potassium and an increase in the extracellular potassium. Fitzgerald et al. (2004) studied the effects of carvacrol and vanillin on *Escherichia coli, Lactobacillus plantarum,* and *Listeria innocua*. At concentrations of 50 mmol/L and 3.3mmol/L of vanillin and carvacrol, respectively, they saw that intracellular potassium levels decreased and the extracellular potassium levels increased. Lambert et al. (2001) looked at oregano essential oil, focusing on the two major components of the oil: carvacrol and thymol. They determined that the addition of 18 µL and 36 µL of oregano essential oil to *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively, caused an increase in extracellular potassium as compared to a control without the oil. Walsh et al. (2003) looked at the ability of thymol and

eugenol to cause potassium leakage in *Escherichia coli* and *S. aureus*. They found that both were able to increase extracellular potassium at concentrations of 500µg/mL and 0.05% v/v, for thymol and eugenol respectively. In two similar experiments by Cox et al. (2000, 2001) the effects of tea tree oil on *E. coli* and *S. aureus* were studied. They found that at 0.25% v/v, one hundred percent of the total cellular potassium was released by *E. coli* within 30 minutes, while only approximately twenty percent was released by *S. aureus* in the same time. Bouhdid et al. (2010) determined the effects of *Cinnamomum verum* on *P. aeruginosa* and *S. aureus*. They found that a concentration of 0.125% (v/v) *Cinnamomum verum* was able to increase extracellular potassium levels in both bacteria. Inoue et al. (2004) examined the terpene alcohols farnesol, nerolidol, and plaunotol for their antimicrobial effects on *S. aureus*. All three compounds increased the extracellular potassium levels when applied to the *S. aureus* at a concentration of 20µg/mL. Togashi et al. (2008) looked at the ability of geraniol and geranylgeraniol to increase the antimicrobial activity of farnesol. They found that when geraniol was added to farnesol in a ration of 2:1, respectively, the ability of farnesol to cause potassium leakage enhanced, while geranylgeraniol inhibited potassium leakage activity of farnesol. These studies indicate that the essential oils they tested act in a similar fashion, with respect to potassium homeostasis. They all act on the membrane to cause the cell to lose the ability to regulate the potassium transfer across the membrane, leading to an out pouring of potassium from the cell. This loss of control is indicative of a loss of viability, and thus is a mode of antimicrobial action for these essential oils.

The release of other cellular components has also been an object of study for those seeking to determine microbial action of essential oils. Release of carboxyfluorescein by *L. innocua* in response to exposure to *Cymbopogon citratus, Ocimum gratissimum,* and *thymus vulgaris* was examined by Nguefack et al. (2004). Carboxyfluorescein diacetate was used to

stain live cells and the cells were then exposed to the essential oils. Loss of fluorescence was taken to be indicative of leakage of carboxyfluorescein, and thus disruption of the cell membrane. All essential oils at concentrations of 1.04×10^4 arbitrary units were shown to cause a decrease in fluorescence as compared to an unexposed control. Xu et al. (2008) looked at the leakage of carboxyfluorescein by *E. coli* after exposure to carvacrol and thymol at different concentrations. They found that both carvacrol and thymol caused an increase in carboxyfluorescein released by *E. coli* cells. Trombetta et al. (2005) examined the release of carboxyfluorescein from large unilamellar vesicles (LUVs) that were exposed to (+) menthol, thymol, and linalyl acetate. They found that (+) menthol and thymol were both effective in causing the release of carboyfluorescein from the LUVs, but linalyl acetate was only slightly effective. Helander et al. (1998) examined the release of fatty acids from *E. coli* cells treated with thymol, carvacrol, (+)-carvone, and *trans*-cinnamaldehyde. Thymol and carvacrol were shown to release a significant amount of fatty acids into the supernatant, while (+)-carvone and *trans*-cinnamaldehyde were ineffective at releasing fatty acids. The results produced by Helander et al. (1998) demonstrated that thymol and carvacrol actually break down the cell membrane. Cox et al. (2001) looked at the release of 280 nm absorbing material by *E. coli* and *S. aureus* after exposure to tea tree oil. Proteins absorb light at 280 nm; therefore release of 280 nm materials is indicative of macromolecular leakage by the cell. *E. coli* and *S. aureus* cells were found to leak 280 nm absorbing material, but at levels lower than potassium leakage. Ifesan et al. (2009), Carson et al. (2002), and Oussalah et al. (2006) all examined the release of 260 nm absorbing material. Nucleic acids absorb light at 260 nm, and therefore their release is indicative of macromolecular leakage by the cell. Ifesan et al. (2009) examined the release of these materials by *S. aureus* strains exposed to a crude extract of a perennial herb, *E. americana*. It was found that there was a peak release of these materials after 8 hours of exposure to 0.25 to

1mg/mL of the extract. After 22 hours of exposure, the absorbance was found to have decreased from the levels seen after 8 hours, most likely due to denaturation of the materials which caused them to become unreactive to the light at 260 nm (Ifesan et al. 2009). Carson et al. (2002) examined the ability of tea tree oil and its components to release 260 nm material from *S. aureus*. They found that a significant amount of material was released by *S. aureus* cells after an hour of exposure to the oil and its components. Oussalah et al. (2006) examined Spanish oregano, Chinese cinnamon, and savory essential oils against *E. coli* O157:H7 and *L. monocytogenes*. They found that at 0.025% (v/v) each essential oil was able to cause an increase in the release of 260 nm absorbing material. These studies indicate that these essential oils and extracts are able to create macromolecular permeability in a variety of bacteria.

The cell membrane helps cells regulate what enters and exits the cell. When ions are no longer being regulated, it indicates that at the very least small pores have formed in the cell membrane. When larger molecules, such as propidium iodide (PI) or *N*-Phenyl-L-napthylamine (NPN), enter the cell without regulation it indicates much larger pores have formed in the membrane and a much higher probability of cell death. Helander et al. (1998) examined the uptake of NPN by *E. coli* and *S.* Typhimurium after exposure to thymol, carvacrol, (+)-carvone, and *trans*-cinnamaldehyde. Thymol and carvacrol significantly increased the uptake of NPN by both bacteria, while (+)-carvone and *trans*-cinnamaldehyde were ineffective. These findings demonstrate that (+)-carvone and *trans*-cinnamaldehyde are unable to form large pores like thymol and carvacrol. *Trans*-cinnamaldehyde has a similar MIC to carvacrol and thymol, according to Helander's study, but it does not function as a large pore former like carvacrol or thymol. Therefore, there must be another mode of action for *trans*-cinnamaldehyde. Fisher and Philips (2009) also looked at NPN uptake by *Enterobacter faecium* and *E. faecalis* after exposure to a citrus oil blend. They found that the oil blend was able to increase the NPN uptake over

two-fold, indicating that the blend is able to cause large pore formation in the bacteria. Cox et al. (2001) examined the uptake of PI by *E. coli* and *S. aureus* after exposure to tea tree oil. They found that nearly 100% of *E. coli* cells showed increased PI uptake, while only around 10% of *S. aureus* cells showed increased PI uptake. Cox's results indicate that there is a possible difference in the effects of tea tree oil on Gram negative and Gram positive bacteria. Bouhdid et al. (2010) found similar results with cinnamon essential oil. *P. aeruginosa* was much more susceptible to the cinnamon oil, in terms of PI uptake, than *S. aureus*. Fitzgerald et al. (2004) looked at the PI uptake by *E. coli*, *L. plantarum*, and *L. innocua* after exposure to vanillin. *E. coli* was seen to show membrane damage after 15 minutes and 60 minutes of exposure, but was seen to show recovery overnight, as the percentage of membrane damage decreased from that seen after 60 minutes. *L. plantarum* exhibited slight damage after 15 and 60 minutes, but did not show the same recovery as *E. coli*, and increased damage overnight. *L. innocua* showed little susceptibility to vanillin. Fitzgerald's results indicates that vanillin does not have the potential to have a Gram negative or Gram positive bias, like tea tree oil or cinnamon essential oil. Although further studies with tea tree oil and cinnamon essential oil need to be carried out to determine if they are more effective at large pore formation in Gram negative bacteria. Based on these studies, large pore formation appears to be a mode of action employed by various essential oils against a range of bacteria.

Decrease in Membrane Potential

Membrane potential is used by the cell to perform actions necessary for life (Ohmizo et al. 2004). Decrease in this membrane potential is indicative of damage to the cell membrane. Some studies have looked at how essential oils affect the cytoplasmic membrane permeability . Bouhdid et al. (2010) used bis-oxonol dye to stain depolarized *P. aeruginosa* and *S. aureus* cells. They found that cinnamon essential oil at MIC levels were able to decrease the membrane

potential of *P. aeruginosa*, but not *S. aureus*. Veldhuizen et al. (2006) used 3,3'-Dipropylthiacarbocyanide iodide($DiSC_35$) to monitor membrane potential. *S. aureus* cells were exposed to carvacrol, *o*-cresol, and 2-amino-*p*-cymene at MIC concentrations. The compounds were found to decrease the membrane potential of the cells within seconds of contact. Ultee et al. (2002) used $DiSC_35$ to look at the disruption of membrane potential of *B. cereus* by carvacrol and cymene. Carvacrol was seen to immediately decrease the membrane potential at concentrations above 0.25 mM, while cymene was able to decrease the membrane potential to a lesser extent at a maximum concentration of 0.5 mM. Fisher and Philips (2009) also used $DiSC_35$ to monitor membrane potential in *E. faecalis* and *E. faecium* after exposure to a 1:1 blend of orange and bergamot essential oils. They found that the blend was able to dissipate the membrane potential within seconds of contact with the cells. These studies show that when an essential oil affects the membrane potential of a cell it is an almost immediate reaction. Loss of membrane potential is adverse to cell survival, but could be a consequence of membrane disruption in the case of carvacrol. Cymene does not show antimicrobial activity, but does show the ability to disrupt membrane potential (Ultee 2002). Ultee et al. (2002) hypothesize that this is due to the leakage of potassium from the cell, but not protons, based on cymene's inability to affect the ATP generation of the cell.

Effects on ATP

Since membrane potential does not always tell the entire story of the mode of action, another target to look at is ATP levels inside and outside the cell. Ultee et al. (2002) determined that cymene, a plant aromatic compound found in cumin and thyme, was unable to increase the ATP levels in the extracellular environment and was unable to decrease the ATP in the intracellular environment. Ultee et al. (1999) also looked at the activity of carvacrol on ATP. Carvacrol eliminated the internal ATP of *B. cereus* cells within 20 minutes of contact at a

concentration of 1 mM. Fisher and Philips (2009) found that their essential oil blend was able to completely dissipate internal ATP in *E. faecalis* and *E. faecium* within 10 minutes. Oussalah et al. (2006) examined the effects of Spanish oregano, Chinese cinnamon, and savory essential oil on the ATP of *E. coli* O157:H7 and *L. monocytogenes*. It was found that all essential oils decreased intracellular ATP, though Chinese cinnamon was less effective than the other two. Savory essential oil was able to increase extracellular ATP levels in both bacteria, while Spanish oregano was only able to increase extracellular ATP in *E. coli* O157:H7 and Chinese cinnamon was not able to increase extracellular ATP in either. Gill and Holley (2004) looked at ATP generation in *Listeria sakei* and *L. monocytogenes.* Both bacteria were treated with eugenol, and *L. monocytogenes* was also treated with cinnamaldehyde. Eugenol was found to inhibit ATP production, but was unable to decrease intracellular ATP. Cinnamaldehyde was found to both inhibit and decrease intracellular ATP. These results were determined by adding the essential oils to the bacteria before or after the addition of 0.25% glucose to energize the cell. Gill and Holley (2004) propose that the mode of action for eugenol relies on inhibiting the cell from using glucose. With cinnamaldehyde showing more diverse effects, Gill and Holley (2004) hypothesize that there may be more than one plausible mode of action. Helander (1998) looked at the effects of carvacrol, thymol, (+)-carvone and *trans*-cinnamaldehyde on the ATP of *E. coli* cells. Carvacrol and thymol were both found to decrease intracellular ATP over a 20 min period, and slightly increase external ATP, but (+)-carvone and *trans*-cinnamaldehyde did not affect the ATP of the cells, internally or externally. Fitzgerald et al. (2004) looked at the effect of vanillin and carvacrol on ATP of *E. coli*, *L. plantarum*, and *L. innocua*. Vanillin was found to be ineffective at reducing intracellular ATP or increasing extracellular ATP in any bacteria. Carvacrol was very effective at decreasing all bacteria's internal ATP, while only increasing external ATP in *E. coli*

and *L. innocua*. From these findings one can conclude that vanillin does not target ATP generation, and that the mode of action must lie elsewhere.

pH Gradient

The ability to maintain a pH gradient is necessary for cell survival, and thus looking at the internal pH or the pH gradient helps to determine if a cell has been severely damaged. Oussalah et al. (2006) and Lambert et al. (2001) both examined the internal pH. Oussalah et al. (2006) found that Spanish oregano, Chinese cinnamon, and Savory essential oils effective at decreasing the internal pH of *E. coli* O157:H7 and *L. monocytogenes*. Lambert et al. (2001) found that oregano essential oil increased the rate of internal pH change in *S. aureus* as compared to an untreated control, indicating a lack of control by the cell to maintain the pH gradient. Ultee et al. (1999,2002), Fitzgerald et al. (2004), Fisher and Philips (2009), and Sikkema et al. (1994) all looked at the pH gradient. Sikkema et al. (1994) looked at liposomes that had been reconstituted with beef heart mitochondrial cytochrome c oxidase, in order to give them a proton motive force. They tested various cyclic hydrocarbons (benzene, toluene, ethylbenzene, o-xylene, and others) and found that all of them dissipated the pH gradient at concentrations related to their membrane partition coefficient. Fisher and Philips (2009) looked at a 1:1 blend of orange and bergamot essential oils. They found that the blend was able completely eliminate the pH gradients of *E. faecium* and *E. faecalis*. Fitzgerald et al. (2004) looked at the effect of vanillin on *L. plantarum*. They found that 100 mmol/L of vanillin was able to dissipate the pH gradient. Ultee et al. (1999, 2002) looked at carvacrol and cymene and found that at 1 mM carvacrol completely dissipated the pH of *B. cereus*, while 2 mM cymene was ineffective. These studies indicate that many oils act to disrupt the membrane in such a way as to make the cell unable to maintain a pH gradient which is essential for generation of a proton motive force, and thus life.

Effect on Respiration

Respiration is an integral part of aerobic metabolism and has potential to be a target for antimicrobial essential oils. Examining the effects of essential oils on respiration can help elicit the mode of action of an essential oil. Bouhdid et al. (2010) looked at the ability of *P. aeruginosa* and *S. aureus* to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) as an indication of respiratory enzyme activity in the presence of cinnamon essential oil. Less than 6% of *P. aeruginosa* cells were able to reduce CTC after a 30 minute exposure to the MIC of the oil. *S. aureus* was found to be more resilient to the respiratory enzyme inhibition by the oil. Roughly 10% of the cells were able to reduce CTC after 60 minutes of exposure to the MIC and 1.5 X MIC of the oil. The results indicate that cinnamon essential oil disrupts the ability of these cells to perform oxidation reduction reactions, and could be a potential mode of action for the oil. Cox et al. (2000) examined the oxygen consumption of *E. coli* and *S. aureus* in the presence of tea tree oil at different concentrations. It was found that 0.5% (v/v) of tea tree oil reduced the oxygen consumption of *E. coli* by 100% and 1.0% reduced the oxygen consumption of *S. aureus* by 60%. Fitzgerald et al. (2004) looked at oxygen consumption in *L. innocua* and *E. coli* when exposed to various concentrations of vanillin. Oxygen consumption was reduced by 19% and 52% in *E. coli* and *L. innocua*, respectively, when exposed to 40 mmol/L of vanillin.

The effect of compound structure on antimicrobial activity

With the knowledge of what cellular processes and structures these essential oils act on, it is important to try and discern what about these compounds allows them to perform these actions in order to better predict antimicrobial activities in other oils. Sikkema et al. (1994) looked at how biological membranes react to cyclic hydrocarbons. They found that the hydrocarbons accumulate in the interior of the membrane causing it to swell. The amount to which a particular hydrocarbon can accumulate in the membrane was found to be directly

related to its membrane partition coefficient. Sikkema et al. (1994) propose that the accumulation of these compounds in the membrane result in changes to the membrane structure and function, thus leading to inhibition or cell death.

Carvacrol is an essential oil that has been shown to have high antibacterial activity and therefore it has been the focus or benchmark for many studies. Some of these studies have looked into what it is about the structure of carvacrol that makes it antimicrobial. (Structures of carvacrol and other essential oil components can be seen in Figure 1). It is a cyclic hydrocarbon, which according to Sikkema et al. (1994) allows it to accumulate in the membrane. It also has a hydroxyl group on the ring which, Ultee et al. (2002) have proposed, gives carvacrol its activity. Ultee et al. (2002) looked at thymol, menthol, carvacrol methyl ether, and cymene in addition to carvacrol. These compounds possess similar structures to carvacrol that only differ in one or two ways. Thymol has the same chemical formula as carvacrol, but the hydroxyl group is in meta position rather than the ortho position. Menthol lacks the benzene ring, and instead has a cyclic hexane ring. Carvacrol methyl ether has the normal hydroxyl group replaced with a methyl ether group. Finally, cymene completely lacks a hydroxyl group. Ultee et al. (2002) found that cymene and carvacrol methyl ether lacked any antibacterial activity. Menthol showed slight inhibitory activity at a concentration of 10 mM for up to 5 hours, but after 24 hours, the bacteria had rebounded to levels seen with only a 0.5 mM concentration of menthol, which showed no inhibition of the bacteria. Thymol showed a very similar pattern of activity to that of carvacrol (Ultee et al.2002). Concentrations above 0.75 mM were completely inhibitory to the bacteria.

Velduizen et al. (2006) found similar results. They examined o-cresol, 3-isoporpylphenol, 2-amino-p-cymene, p-cymene, and 3,4-dimethylcumene in comparison to carvacrol. They found that *p*-cymene (same as cymene) and 3,4-methylcumene exhibited no antibacterial activity. In 3,4-methylcumene the hydroxyl group of carvacrol is replaced with a methyl group, indicating

that the hydroxyl group is an important part to the antibacterial activity. The compound 3-isopropylphenol, which lacks the methyl group on the ring, was found to have an MIC approximately 1.5 times that of carvacrol. O-cresol, which lacks the isopropyl group of carvacrol, was found to have an MIC approximately 2 times that of carvacrol. The MIC of these two carvacrol related compounds indicates that the R groups do play a small role in the antibacterial activity of carvacrol, though not as much as the hydroxyl group. The compound 2-amino-p-cymene has an amino group in the same position as the hydroxyl group of carvacrol, but the rest of the structure remains the same. The MICs of 2-amino-p-cymene were approximately 4 mM higher than that of carvacrol, and had the highest MIC of all antibacterially active compounds tested. The compound 2-amino-p-cymene shows antibacterial activity, indicating that the hydroxyl group is not essential for antimicrobial activity, although it appears to enhance antibacterial activity.

Arfa et al. (2006) also examined how the structure of carvacrol affects its antibacterial activity. They compared carvacrol to carvacrol methyl ether, carvacrol acetate, eugenol, and menthol. Carvacrol methyl ether and carvacrol acetate were not found to show any antibacterial activity. Both of these compounds have substitutions at the hydroxyl group of carvacrol respective of their names, indicating that these groups cannot convey the same activity as the hydroxyl group of carvacrol. Eugenol, which is another aromatic compound like carvacrol, has a hydroxyl group and an ether group which are para and ortho, respectively, to a 3 carbon chain that ends in a double bond. Eugenol was found to exhibit a lower antibacterial activity than carvacrol, but was not inactive like carvacrol acetate and carvacrol methyl ether. Arfa et al. (2006) only determined MICs for their compounds, so it is difficult to say if the antibacterial action of eugenol is the same as carvacrol, although it can be said that not all antibacterial essential oil compounds must have a structure similar to that of carvacrol in order to possess

activity. Similar to Ultee et al. (2002), Arfa et al. (2006) found menthol to possess very little antibacterial activity, which supports the theory that the benzene ring is important for antibacterial activity. With the added data from the carvacrol derivatives, it can also be said that the benzene ring is important, but only for those compounds that possess a hydroxyl group or an amino group, as was the case with 2-amino-p-cymene in the study by Velduizen et al. (2006).

Gill and Holley (2004) looked at the two aromatic compounds eugenol and *trans*-cinnamaldehyde. Both compounds were found to exhibit antibacterial activity, although eugenol had a lower MIC. *Trans*-cinnamaldehyde, which contains an aromatic ring with a 3 carbon aldehyde chain containing a double bond at the second position, was able to decrease internal ATP of energized cells and inhibit ATP increases of non-energized cells of *L. monocytogenes*. Eugenol, which was described earlier, was able to inhibit ATP increases of non-energized cells, but was unable to reduce ATP levels of energized cells. Their results indicate that *trans*-cinnamaldehyde could be acting via membrane disruption without the need of a hydroxyl group like carvacrol. It is difficult to compare carvacrol and *trans*-cinnamaldehyde since they were not used in the same study, but if one extrapolates the results from Gill and Holley (2004) and Arfa et al. (2006), one can make the assumption that *trans*-cinnamaldehyde has lower antibacterial activity than carvacrol. This is due to the fact that *trans*-cinnamaldehyde was found to have lower antibacterial activity than eugenol and that eugenol was found to have lower antibacterial activity than carvacrol. Therefore, *trans*-cinnamaldehyde probably causes membrane disruption to a lesser extent than carvacrol due to its lack of a hydroxyl group. Bouhdid et al (2010) studied cinnamon essential oil, which was mainly composed of *trans*-cinnamaldehyde. They found that the essential oil caused leakage of potassium ions from *P. aeruginosa* and *S. aureus*, which furthers the idea that *trans*-cinnamaldehyde is a membrane disruptor. Helander et al. (1998) also looked at *trans*-cinnamaldehyde, but found it was unable to induce ATP leakage. This

contradictory result is most likely due to the concentration difference used in the two studies. Helander et al. (1998) used 2 mM, while Gill and Holley (2004) used 40 mM.

Conclusion

With the knowledge of current technologies employed to reduce bacteria and bacterial pathogens, like *E. coli* O157:H7, and where they are used during the slaughter process, it is evident that most beef processing plants are leaving out the chill stage as a potential antimicrobial intervention point. Few have looked into this point of intervention, and currently no one is looking to essential oils to fill the role of antimicrobial at this stage. With the understanding of how essential oils and their components work to kill bacterial cells, it is possible to select an essential oil that could be applied onto beef carcass surfaces during the chill stage of processing. Research should be undertaken to explore the efficacy of applying essential oils during the chill stage of processing. This would give processors an additional point, closer to the end of processing to kill potential pathogens and prevent them from reaching the consumer.

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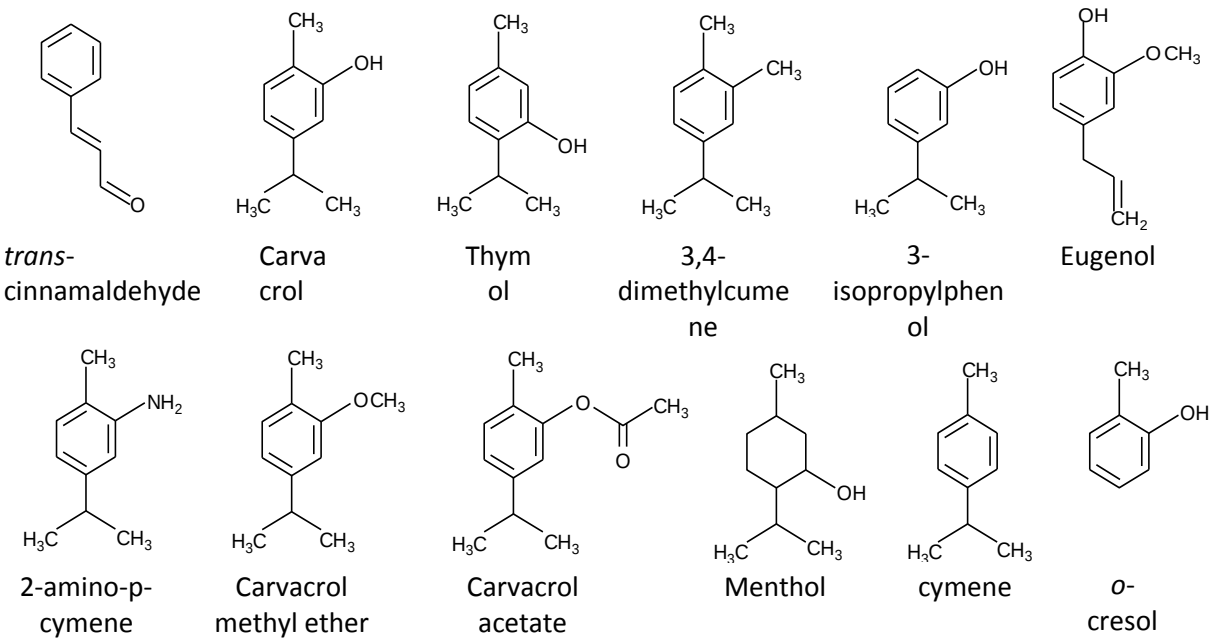

Figure 1. Chemical structures of essential oil components and carvacrol derivatives.

Inhibition of *Escherichia coli* O157:H7 Beef Isolates by Cold Pressed Terpeneless Valencia Orange Oil at Various Temperatures

Inhibition of *Escherichia coli* O157:H7 Beef Product Isolates by Cold Pressed Terpeneless Valencia Orange Oil at Various Temperatures

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Abstract

This study was undertaken to identify antimicrobials for use during chilling in beef processing, including periods of temperature abuse. Naturally occurring plant essential oils have been shown to be antimicrobial. Cold pressed terpeneless Valencia orange oil was examined in combination with various temperatures (37°C, 10°C, and 4°C) to determine its antimicrobial activity against three strains of *Escherichia coli* O157:H7 recovered from beef products. The strains were tested using a ninety-six well microtiter plate method with TSB+0.15% agar and a growth indicator, 2, 3, 5-triphenyl tetrazolium chloride. Serial, two fold dilutions gave concentrations of oil ranging from 25 to 0.2% or 10 to 0.1%. Plates were incubated statically at 37, 10, or 4°C for times up to 48 hours. After 6 hours at 37°C, all strains were inhibited at oil concentrations ranging from 0.6% to 0.2%, with a mean of 0.4±0.01%. At 10°C, all strains were shown to be inhibited at concentrations ranging from 1.4 to 0.9%, with a mean of 1.1 ± 0.2%, after 6 hours. At 4°C, all strains were shown to be inhibited after 6 hours at concentrations ranging from 4.6 to 2.3%, with a mean of 3.5 ± 2.1%. After 24 hours at 4°C the strains were inhibited at concentrations ranging from 1.0% to 0.7% with a mean of 0.8 ± 0.3%. These ranges appear to be the result of effects from the variable nature of a complex media and an antimicrobial that presents potential multiple mechanisms for inhibition of *E. coli* O157:H7 at refrigeration temperatures.

Introduction

There are an estimated over 170,000 cases of shiga toxin producing *E. coli*, (STEC) in the United States each year. Of these cases, approximately 2,400 result in hospitalization (Scallan 2011). The estimated economic cost of these STEC infections was approximately 993 million dollars in 2009 (Health-Related Costs 2010). This cost does not include the expense for companies who are required to recall the foods associated with the foodborne infections. A major source of *E. coli* infections is ground beef. The most recent multistate outbreak of E. coli O157:H7, as reported by the Centers for Disease Control and Prevention (CDC) (2010), required National Steak and Poultry to recall approximately 248,000 pounds of beef product. Processing of cattle includes removal of the hide, evisceration, trimming to remove visible contamination, carcass washing, and ultimately chilling (Beef Industry Food Safety Council 2009). Most intervention steps occur prior to chilling and therefore any O157:H7 contamination still present at the chilling step has potential to be passed on to the consumer. The fact that O157:H7 outbreaks are still occurring indicates additional hurdles during or post chilling are required to prevent O157:H7 from reaching consumers.

Essential oils have been in use for centuries as pharmaceuticals, and are currently used mainly for fragrances and flavors (Van de Braak and Leijten 1999). In the late 19th century, the antimicrobial properties of essential oils were first explored (Burt 2004). Although essential oils are mainly used as flavor and fragrance, there has been an emphasis on the possible role of essential oils as antimicrobials (Cowan 1999, Holley and Patel 2005). Deans and Ritchie (1987) examined the antibacterial properties of fifty different essential oils against 25 genera of bacteria. They found variation in effectiveness among oils and among strains, showing that there are few, if any, antibacterial essential oils that exhibit a constant level of antimicrobial activity against a broad spectrum of genera. Given this variability, there is a need for more

research into essential oils and their antimicrobial applications under specific environmental conditions to optimize their application in food systems.

The use of essential oils to inhibit and kill bacteria is generally examined at human physiological temperatures (37º C) or optimal growth temperatures for the organism. However, these studies only give us an understanding of how the bacteria react when placed under optimal conditions for growth, but do not provide understanding as to how the bacteria would react when under actual conditions at which foods are stored. Therefore, it is important to include temperature as an additional variable when determining antibacterial activities of essential oils.

The chilling stage of processing seeks to rapidly bring the freshly slaughtered carcasses to 4°C. Once this temperature is reached, retailers attempt to maintain this temperature as a part of a "cold chain" until the consumer stores the hamburger in their home refrigerator. The recommended operating temperature for these appliances is 4°C, though depending on maintenance this temperature can fluctuate up to and above 10°C (Kosa 2007). Similar to home refrigerators, chillers are not always operating at the optimal temperature, therefore it is appropriate to look at temperature abuse situations. A commonly identified abuse temperature is 10°C (Berdholt 1999, Maca 1999, Cortesi 1997, and Okereke 1991), and thus was the temperature focused on this study. It was the aim of this study to determine the minimum inhibitory concentration (MIC) of an essential oil, cold pressed terpeneless Valencia orange oil at 10 and 4°C against three strains of *E. coli* O157:H7.

Materials and Methods

Materials and Bacterial Strains

Commercially available cold pressed terpeneless Valencia orange oil (Orange CP Val Terpeneless FAB 968611) was obtained from Firmenich (Lakeland, FL). Three O157:H7 strains of

Escherichia coli, isolated from beef, were used, *E. coli* 933, 505B and American Type Culture Collection (ATCC) 43895. *E. coli* strains 933 and 505B were kindly donated by Michael P. Doyle at the University of Georgia. The strains were grown in one of the following ways: in Tryptic Soy Broth (BD, Franklin Lakes, NJ, USA) with 0.15% granulated agar (BD, Franklin Lakes, NJ, USA) at 37°C for 18-24hrs, or grown in TSB at 37°C for 18-24hrs, centrifuged at 7000 rpm for 10min, and resuspended in 10mL of TSB + 0.15% agar. The inclusion of 0.15% granulated agar in the growth medium allowed the oil to better incorporate into the test medium (Friedly and others 2009).

Determination of Minimum Inhibitory Concentration (MIC) at 10°C

The MIC method used was a modification of the 96-well microtiter plate method used by Friedly and others (2009). A ninety-six well plate (TPP Zollstrasse 155 8219 Trasadingen, Switzerland) was prepared by the addition of 100 µL of TSB + 0.15% agar with 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) to all wells. One hundred microliters of cold pressed terpeneless Valencia orange oil were added to wells A1-9, and serial two fold dilutions were carried out down each column from row A to row H. Wells A10-12, B10-12, and C10-12 served as positive controls and contained only TSB + 0.15% agar + TTC and culture. Wells D10, 11, and 12 served as negative controls and contained only 25% cold pressed terpeneless Valencia orange oil in TSB+ 0.15% agar + TTC. The two-fold dilutions resulted in concentrations of cold pressed terpeneless Valencia orange oil ranging from 25% to 0.2%. One hundred microliters of *E. coli* 933 was added to wells in columns 1, 4, 7 and 10; *E. coli* 505B to wells in columns 2, 5, 8 and 11; and *E. coli* 43895 to wells in columns 3, 6, 9 and 12 (Figure 1). After the plates were prepared, they were incubated statically at 10°C for time intervals of 1, 2, 3, 4, 5, and 6 hours. Temperature was checked via mercury in glass thermometer at the beginning and end of each time interval. At each sampling time, 10 µL aliquots from each well were line streaked onto Trypticase Soy Agar (TSA). The microtiter plate was then left at room

temperature to allow the bacteria additional time to reduce the TTC to TPF and produce a red pigment indicating growth. The TSA plates were incubated for 24 hrs at 37°C, after which time growth observations were recorded. The experiment was repeated four times. An additional study was performed in the same manner as detailed above with two repetitions, except the plates were incubated at 37°C for 48 hours.

Determination of Minimum Inhibitory Concentration (MIC) at 37°C and 4°C

Ninety-six well plates were prepared by adding 100 µL of TSB + 0.15% agar + TTC to each well, except wells A1-10. To wells A1-10, 200 µL of 20% oil + TSB + 0.15% agar were added. Using a multichannel pipette, 100 µL of oil suspension in the first wells were transferred into the next wells for a 1:2 dilution and this was repeated down the plate. This resulted in concentrations from 10 to 0.1% of cold pressed terpeneless Valencia orange oil. Wells in columns 11 and 12 were used for positive controls and contained only culture and TSB + 0.15% agar + TTC. Column 10 served as a negative control. After dilutions were made, 100 µL of each culture were added to respective wells. Plates were incubated statically at either 37°C or 4°C for 6hrs. After incubation, plates were removed and 10 µL from each well and were spotted onto TSA plates. The ninety-six well plates were then placed back into 37°C or 4°C incubation for an additional 6hrs. After second incubation, plates were removed and plated as previously described. The Ninety-six well plates were placed back into incubation for an additional 12hrs and then plated as previously described. All TSA plates were incubated at 37°C for 24hrs. After 24hrs, growth observations were made and the respective MIC was determined for each culture.

Statistical Analysis

The data were compiled in Excel 2007. The repetitions were averaged and means were compared using a two sample Student-T test. Differences were considered significant when $p<0.05$.

Results and Discussion

At 37°C, all three strains reached MICs after the first 6 hours of incubation that were statistically not different, ($p>0.05$), from the MICs observed at 24 hours (Table 1). *E. coli* strain 933 was found to have the lowest MIC, 0.2 ± 0.0%. *E. coli* stains 505B and 43895 had higher MICs, 6.0 ± 0.0% and 0.5 ± 0.1% respectively. After 24 hours at 37°C, the MICs for each strain were significantly different from each other, ($p<0.05$).

After one hour at 10°C, all three strains exhibited MICs that were determined to be statistically indistinguishable ($p<0.05$) (Table 1). After two hours, *E. coli* 43895 had an MIC of 0.8±0.03 which was significantly lower than *E. coli* 933 which exhibited an MIC of 2.2±0.6 at a p-value of 0.05. *E. coli* 505B was not significantly different from either of the other two strains after two hours, ($p>0.05$). All three strains' MICs were approximately 1.0% and were, again, statistically indistinguishable after three hours. At the four hour mark, *E. coli* 43895 had an MIC, 0.7±0.04, which was significantly lower than that of *E. coli* 505B, 0.9±0.1. The MIC for *E. coli* 933 was statistically indistinguishable from the other two strains. At hour five, the MICs for each strain were statistically indistinguishable. After six hours, all MICs were approximately 1% and were statistically not different, ($p>0.05$).

At hour four, *E. coli* 933 had an MIC of 1.0±0.2 which was significantly lower than the MIC at hour two, 2.2±0.6. The MICs for the other time periods did not differ from each other. The MICs at hours three, four, and six (approximately 1%) for *E. coli* 505B were also the same, but were significantly different from hour one, which was 2.1±0.4. The MICs for *E. coli 505B* at

hours two and five did not differ significantly from the MICs at other time points. For *E. coli* 43895, the MICs at hours two, 0.8±0.03, and four, 0.7±0.04, were significantly lower than the MIC at hour one, which was 1.4±0.2. The MICs of *E. coli* 43895 for hours three, five, and six were not significantly different from the MICs at other time periods.

When the strains were exposed to the oil at 4°C, they exhibited a decline in MIC values over the 24 hour period of sampling, to concentrations nearing the MICs observed at 37°C and 10°C. For all strains, the MIC observed at 24 hours was significantly lower, ($p<0.05$), than that observed at 6 hours. *E. coli* stain 933 had an MIC of 2.3 ± 0.4% after 6 hours and an MIC of 0.7 ± 0.1% after 24 hours. *E. coli* strain 43895 had an MIC of 3.6 ± 0.7% after 6 hours and an MIC of 0.7 ± 0.1% after 24 hours. *E. coli* strain 505B had an MIC of 4.4 ± 0.8% after 6 hours and an MIC of 1.0 ± 0.2% after 24 hours.

When comparing the 37°C results to the 4°C results, the data indicate that there was a temperature effect. For each strain, the MIC after 24 hours at 4°C is higher than that at 37°C, though the two lowest MICs observed at 4°C, 0.7 ± 0.3% and 0.7 ± 0.1%, are not greatly different from the highest MIC observed at 37°C, 6.0 ± 0.0%. At each time point under the 4°C conditions, strains 933 and 43895 had MICs that were not significantly ($p>0.05$) different from each other. However, under the 37°C conditions, these same two strains differed significantly ($p<0.05$).

The data indicate that there is a temperature effect on the antimicrobial activity of cold pressed terpeneless Valencia orange oil, but there is also a time effect. At the 6 hour time point, 37°C MICs were the lowest, followed by 10°C and 4°C. It took twice as long for the oil to reach MICs values near those seen after 6 hours at 10°C for strains 933 and 43895, and the oil was not able to reach the MIC values seen for 37°C, even after 24 hours. Although, increasing the time of exposure of those strains to the oil at 10 and 4°C could decrease the MIC values to those seen at 37°C, but this requires further study. Strain 505B exhibited more resistance to the oil at 4°C, in

that the MIC values were higher than those for the other two strains and that it took longer to reach MIC values seen after 6 hours at 10°C. At 37°C, 505B was also the most resistant, based on MIC, but not at 10°C where all the strains were not significantly different after 6 hours ($p<0.05$).

There are previous studies that have looked at the antibacterial activities of essential oils under freezing and refrigerated conditions. Studies have investigated temperatures from 0°C (Skandamis and Nychas 2000) to 16°C (Valero and Salmerón 2003), and various temperatures in between: 4, 5, 7, 8, 10, 12 and 15°C (Burt and Reinders 2003, Solomakos and others 2008, Friedman and others 2004, Skandamis and Nychas 2000, Bagamboula and others 2003, Oussalah and others 2004, Valero and Salmerón 2003). Burt and Reinders (2003) found a slight decrease in minimum inhibitory/bactericidal concentration of oregano and thyme oils against *E. coli* O157:H7 in an agar stabilized broth solution when kept at 10°C, as compared to 37°. In a study by Skandamis and Nychas (2000), it was seen that at a concentration of 1.4% oregano essential oil and a pH of 4.5 that *E. coli* O157:H7 NCTC 12900 was reduced by nearly 3.5 logs in less than one day when kept at 10°C. Solomakos and others (2008) examined the effect 0.6% thyme essential oil at 10°C on *E. coli* O157:H7 in minced beef. They found that it was effective in inhibiting growth of *E. coil* 933 and 932 for six days, after which time they saw an outgrowth of approximately 2 logs over the following six days. Although there was an eventual outgrowth after six days, the oil was still an effective inhibitor for the first six days of storage at 10°C and kept the final counts 1 log below the control counts. At 4°C, the 0.6% thyme essential oil did not perform any better than the control over the 12 day study, but a combination of 0.6% thyme essential oil and nisin (500 or 1000 IU/g) did reduce the counts by 1 log over the initial two days and inhibited any out growth during the remaining 10 days. Friedman and others (2004) examined the antibacterial properties of plant essential oils and their components against a single strain of *E. coli* O157:H7 and *S. enterica* in apple juice. They examined carvacrol,

cinnamaldehyde, citral, and thyme oil in a time and temperature study where they looked at the effects at 5, 60, and 120 minutes and at 4, 21, and 37°C. For carvacrol, they found it to be more active against *E. coli* and *S. enterica* at 37°C than at the lower temperatures. For cinnimaldehyde and citral, there was not a great temperature effect against *E. coli*, most likely due to the relative inactivity of the compounds against the bacterium. There was a temperature effect against *S. enterica* for cinnimaldehyde and citral, both were more active at 21 and 37°C, than at 4°C. Thyme oil was more active against *S. enterica* at 37°C after 2 hours than at 21 or 4°C after the same amount of time. Against *E. coli*, thyme oil was found to be more active as time progressed, but not as temperature increased. The results of Friedman and others are similar to ours with respect to decrease in activity at colder temperatures, though they claim a threefold decrease in activity at colder temperatures, while we only saw around a threefold decrease for only one of our strains and less than a twofold decrease for the other two strains.

The current use of lactic acid sprays to treat carcasses for *E. coli* O157:H7 works due to the ability of lactic acid to disrupt and disintegrate the outer-membrane of Gram-negative bacteria via acidification in the dissociated form and additional activity in the undissociated acid form (Alakomi 2000). One study showed that at 0, 5 and 10°C pH alone was unable to reduce *E. coli* O157:H7 to an undetectable level, but 15°C caused a reduction to undetectable levels within 16 days (Skandamis and Nychas 2000).This shows that low temperatures can decrease the action of acidification and therefore render acidification an ineffective intervention method for the chill step of beef processing. Our study demonstrates that there is a decrease in activity at refrigeration temperatures of cold pressed terpeneless Valencia orange oil when used against *E. coli*, but in our case we found that the oil was able to inhibit the pathogen's growth to an undetectable level at refrigeration temperatures.

Friedly and others (2009) found similar results for *Listeria*. They determined an MIC of 0.55% cold pressed terpeneless Valencia orange oil required to inhibit the growth of *Listeria* spp. at 37°C. Friedly and others also examined the oil using disc diffusion, and found that it produced an average inhibition zone of 25.6 ± 3.7mm against *L. monocytogenes* and an average inhibition zone of 27.0 ± 4.4mm against *L. innocua*. In contrast, O'Bryan and others (2008) found that cold pressed terpeneless Valencia orange oil showed little antimicrobial activity against various strains of *Salmonella*. Using a disc diffusion assay, O'Bryan and others found that the greatest activity was seen against *Salmonella* Enteritidis and was able to produce a zone of inhibition of 12.7 ± 2.3mm. For the other strains, this zone was approximately 8mm or less. Nannapaneni and others, in 2008, found that cold pressed terpeneless Valencia orange oil was more inhibitory to *E. coli* O157:H7 than what was seen by O'Bryan and others against *Salmonella*. They observed that, for 12 strains of O157:H7 tested, cold pressed terpeneless Valencia orange oil produced zones of inhibition of at least 9.5 ± 0.7mm, with the largest zone being 14.5 ± 0.7mm. The difference in activity between what was seen by Friedly and what was reported by O'Bryan and Nannapaneni could be due to the greater antimicrobial activity of essential oils against Gram-positive bacteria (Burt 2004). This effect of essential oils against Gram-positive bacteria was shown by Fisher and Phillips (2006), when they examined the antibacterial activity of citrus oils against Gram-positive and Gram-negative bacteria. They observed that *L. monocytogenes*, *B. cereus*, and *S. aureus* were significantly more susceptible, overall, to the oils tested than *E. coli* O157 and *C. jejuni*. While cold pressed terpeneless Valencia orange oil showed activity against *Salmonella* and *E. coli*, these studies did not determine a minimum inhibitory concentration, and therefore it is difficult to compare whether or not there is a large difference in MIC between gram positive bacteria and gram negative bacteria with these studies. Our data indicate that, at

37°C, there is little difference in MIC. We determined that *E. coli* 505B had the highest MIC of 0.6%, which was only 0.05% higher than what was seen by Friedly and others.

Lis-Balchin and Deans (1997) found that there was substantial difference in antimicrobial activity of the individual essential oils against a range of different strains of *L. monocytogenes*. Our study was also subject significant variation between strains (Table 2). Nannapaneni and others (2008) saw significant variation in antibacterial activity for cold pressed terpeneless Valencia orange oil against different isolates and mutant strains of *E.* coli O147:H7 when they were exposed to the oil for 48 hours at 37°C. They found that the strain most susceptible, which was significantly more susceptible that the other strains tested ($p<0.05$), to cold pressed terpeneless Valencia orange oil was a mutant strain that only produced SLT toxin I (ATCC 43890). They also determined that a toxin negative mutant (ATCC 43888) had the same susceptibility as the strain that produced both toxins (ATCC 43895). ATCC Strain 43889, which only produces SLT II, was found to have the same susceptibility as strain 505B. This data indicate that toxin production does not play a significant role in susceptibility to the orange oil. Of the twelve *E. coli* O157:H7 strains tested, only strain 932 and ATCC 43890 were significantly different from the rest. Strain 932 was found to be significantly less susceptible, $p<0.05$. In the current study, strain 933 was found to be significantly more susceptible to the oil, as compared to strains 505B and ATCC 43895, when exposed to the oil for 24 hours at 37°C. This result is different from Nannapaneni and others (2008) finding, but could be due to the difference in the methods used to determine antibacterial activity. The results of this study and these others indicate the need to screen multiple strains of the same species or serotype when examining essential oils.

Conclusion

In the beef industry, carcasses are chilled to temperatures close to or at 4°C within 24 hours. Our results indicate that a solution of 1% cold pressed terpeneless Valencia orange oil could be used as an additional intervention against *E. coli* O157:H7 at the chilling stage of processing, and will remain effective if the carcasses are subjected to temperature abuse. Further research into additional temperatures and strains are needed to verify these claims.

Acknowledgement

Funded by The Beef Checkoff.

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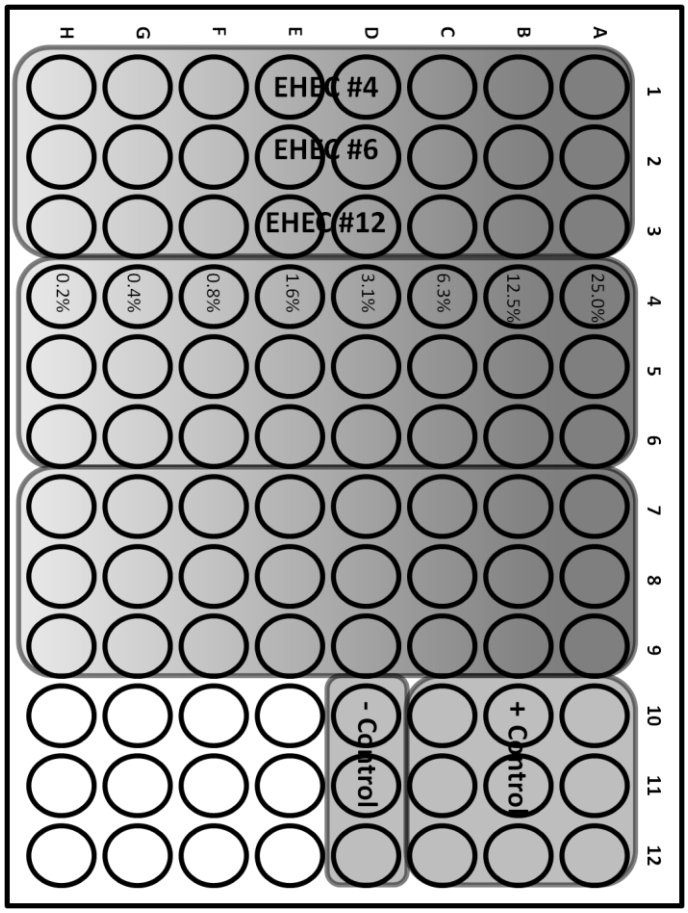

Figure 1. 96-well microtiter plate design for MICs at 10°C with strain locations and cold pressed terpeneless Valencia orange oil concentrations in percentages. EHEC #4, *E. coli* 9 33; EHEC #6, *E. coli* 505B; EHEC #12, *E. coli* 43895.

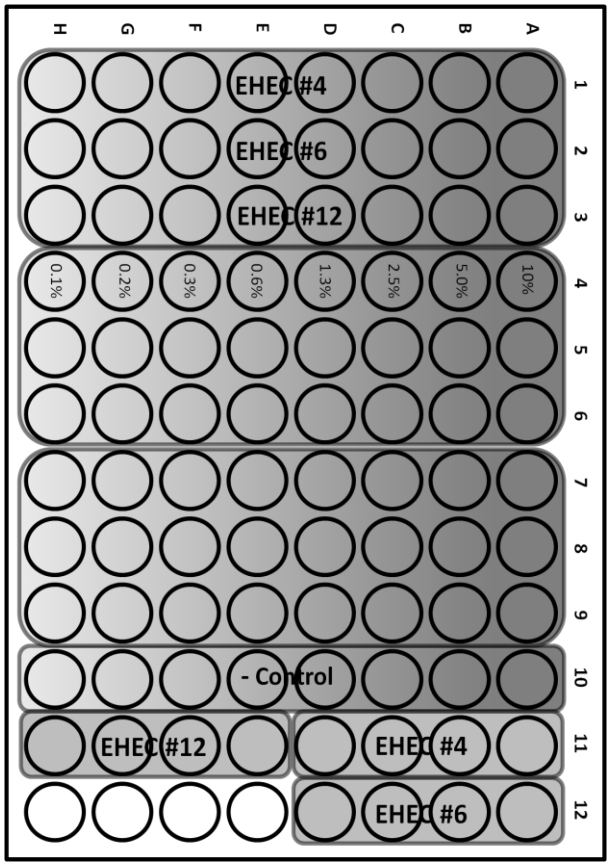

Figure 2. 96-well microtiter plate design for MICs at 37°C and 4°C with strain locations and cold pressed terpeneless Valencia orange oil concentrations in percentages. EHEC #4, *E. coli* 933; EHEC #6, *E. coli* 505B; EHEC #12, *E. coli* 43895.

Table 1. Minimum inhibitory concentrations of cold pressed terpeneless Valencia orange oil at 37°C.

Time (hr)	*E. coli* 933 (%)		*E. coli* 505B (%)		*E. coli* 43895 (%)	
6	0.2±0.0[1]	a[2]A[3]	0.6±0.0	a B	0.5±0.0	a C
12	0.2±0.0	a A	0.6±0.0	a B	0.5±0.0	a C
24	0.2±0.0	a A	0.6±0.0	a B	0.5±0.0	a C

[1]Mean±SEM

[2]Lower case letters indicate statistical differences within columns ($p<0.05$)

[3]Upper case letters indicate statistical differences within rows ($p<0.05$)

Table 2. Minimum inhibitory concentrations of cold pressed terpeneless Valencia orange oil at 10°C.

Time (hr)	*E. coli* 933 (%)		*E. coli* 505B (%)		*E. coli* 43895 (%)	
1	6.0±2.6 [1]	ab[2] A[3]	2.1±0.4	a A	1.4±0.2	a A
2	2.2±0.6	a A	2.3±0.9	ab AB	0.8±0.03	b B
3	1.2±0.2	ab A	1.0±0.1	b A	1.0±0.2	ab A
4	1.0±0.2	b AB	0.9±0.1	b A	0.7±0.04	b B
5	2.5±1.0	ab A	3.3±1.3	ab A	1.1±0.5	ab A
6	1.4±0.5	ab A	0.9±0.1	b A	1.0±0.2	ab A

[1]Mean±SEM

[2]Lower case letters indicate statistical differences within columns ($p<0.05$)

[3]Upper case letters indicate statistical differences within rows ($p<0.05$)

Table 3. Minimum inhibitory concentrations of cold pressed terpeneless Valencia orange oil at 4°C.

Time (hr)	*E. coli* 933 (%)		*E. coli* 505B (%)		*E. coli* 43895 (%)	
6	2.3±0.4[1]	a[2]A[3]	4.4±0.8	a B	3.6±0.7	a AB
12	1.3±0.4	ab A	3.6±0.9	a B	1.3±0.2	b A
24	0.7±0.1	b AB	1.0±0.2	b B	0.7±0.1	c A

[1]Mean±SEM

[2]Lower case letters indicate statistical differences within columns ($p<0.05$)

[3]Upper case letters indicate statistical differences within rows ($p<0.05$)

Efficacy of a Membrane Filtration Method for Determining Minimum Inhibitory Concentrations of an Essential Oil

Efficacy of a Membrane Filtration Method for Determining Minimum Inhibitory Concentrations of an Essential Oil

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Short Title: Membrane Filtration of an Essential Oil

Abstract

The relative efficiency of an essential oil as a bacteriostatic or bactericidal agent was determined by minimum inhibitory concentrations (MICs). MICs are commonly determined via broth dilution method or by measuring clearing on agar. These methods rarely include a neutralization step to prevent the continued action of the antimicrobial. Essential oils contain many potentially antimicrobial elements, and finding a neutralizer to inactivate these multiple elements can be difficult. A novel method, combining a micro-broth dilution method and membrane filtration to physically remove the oil, was developed to evaluate the activity of cold pressed terpeneless Valencia orange oil against multiple species of bacteria. A ninety-six well microtiter plate method was used to expose the cells to the oil, followed by a transfer of the oil and bacteria mixture to a ninety-six well filter plate. After an initial filtration by centrifugation, the contents of the filter plate wells were washed twice with TSBYE + 0.5% Tween 20 to separate the oil from the bacteria. The bacteria were resuspended in TSBYE containing a growth indicator and transferred to a sterile 96 well microtiter plate to determine MICs. *Escherichia coli* O157:H7 was inhibited at a concentration of 0.5±0.0%, *Listeria monocytogenes* at 0.5±0.0%, *Staphylococcus aureus* at 0.31±0.13%, *Salmonella* Typhimurium at 0.31±0.13%, *Shigella sonnei* at 0.75±0.29%, *Yersinia enterocolitica* at 0.31±0.13%, *Enterococcus faecalis* at 0.63±0.25%, *Bacillus cereus* at 0.44±0.13%, and *Pseudomonas aeruginosa* exhibited complete resistance to the oil. The MICs found were not significantly greater than those found in previous studies, indicating that removal of the essential oil by membrane filtration is not required for determination of MICs for essential oils.

Introduction

The main methods for determining minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of essential oils and essential oil components are broth dilution, agar diffusion, and disc diffusion (Kim et al. 1995, Tassou et al. 2000, Fernandez-Lopez et al. 2005, O'Bryan et al. 2008, Nannapaneni et al. 2008, and Friedly et al. 2009). These methods typically do not employ a neutralization step that would quench the antimicrobial activity of the antimicrobial being tested when MICs and MBCs are observed. In order to make an accurate assessment of an antimicrobial's effect on a bacterium, an additional step is needed to allow injured, but still viable, cells a chance to recover. This recovery step must include media devoid of the antibacterial compound or neutralizers to inactivate the antimicrobial compound (Meers and Churcher 1974, Prince et al. 1975, and Dey and Engley 1983). It is a well-established fact that essential oils are made up of many different compounds (Kubo et al. 2004, Burt et al. 2005, and O'Bryan et al. 2008), so finding a broad spectrum neutralizer to inactivate all of the potential antimicrobial compounds becomes problematic. Since there is not a universal neutralizer for all essential oils, then a logical approach is to separate the essential oils from the bacteria at the end of the timed exposure period.

Meers and Churcher (1974) and Prince et al. (1975), both looked at membrane filter techniques. Meers and Churcher (1974) used a membrane to filter volumes of treated cells in order to separate out the cells from antimicrobial drug treatments. The cells retained on the filters were washed, then placed on agar, and incubated for 24 hours. Plates were then counted to determine viable cells. Prince et al. (1975) used a different approach. Cells were first placed on the membrane and then exposed to a disinfectant treatment for two and half or eight minutes. The disinfectant was then filtered away and the membrane was washed. The membrane was then transferred to agar and incubated for 24 to 48 hours, after which counts

were made to determine antibacterial activity. Both techniques found that the antimicrobial compounds were able to be effectively removed from the cells via membrane filtration.

Membrane filtration offers the ability to remove antibacterial compounds without the need for neutralizers, especially when an adequate neutralizer is not available (Bergan and Lystad 1972). This aspect of membrane filtration indicates its promise for the use in essential oil MICs and MBCs. Therefore, the aim of this study was to determine the efficacy of membrane filtration in the determination of minimum inhibitory concentrations for cold pressed terpeneless Valencia orange oil against several species of bacteria.

Materials and Methods

Bacterial strains

Nine bacterial strains were used for this study: *Escherichia coli* O157:H7 (ATCC 43888), *Listeria monocytogenes* (USFDA), *Staphylococcus aureus* (ATCC 25923), *Salmonella* Typhimurium (ATCC 14028), *Shigella sonnei* (ATCC 25931), *Yersinia enterocolitica* (ATCC 23715), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), and *Pseudomonas aeruginosa* (ATCC 27853*).* Cultures were grown in 10mL of TSB + 0.5%YE in a 15 ml centrifuge tube and incubated (GCA/Precision Scientific, model 6M) at 37°C without shaking for 24 hours. The cultures were then centrifuged at 6000-8000 rpm for 10 min. The supernatant was poured off and the pellet was resuspended in 10 ml of 20 mM PBS. A second wash was performed and the final pellet was resuspended in TSB + 0.5%YE + 0.5% Tween 20 and diluted to give resulting bacterial concentrations of approximately 10^6 CFU/mL.

Essential Oil

Commercially available cold pressed terpeneless Valencia orange oil (Orange CP Val Terpeneless FAB 968611) was obtained from Firmenich (Lakeland, FL). The initial oil suspension was made by adding 0.2 mL of cold pressed terpeneless Valencia orange oil to 10 ml of tryptic

soy broth with 0.5% yeast extract (TSB + 0.5%YE) and 50 µL of Tween 20 in a 10 ml centrifuge tube, giving a 2% oil and a 0.5% Tween 20 concentration.

Methods

Two hundred µL of the oil suspension was added into the first wells (column 1) of a sterile, 96 well tissue culture plate. In the following wells (columns 2 through 6) 100 µL of TSB + 0.5%YE + 0.5% Tween 20 were added to each well. With a multichannel pipette, 100 µL of oil in the first wells was transferred into the second wells for a 1:2 dilution and followed by additional 1:2 dilutions. The final oil concentrations were 1%, 0.5, 0.25, 0.13, 0.63, and 0.03. For negative controls, 100 µL of TSB + 0.5%YE + 0.5% Tween 20 was added to row G, to bring final volumes up to 200 µL. In triplicate, 100 µL of each 10^6 culture was added to the various concentrations of oil (Figure 1.). An adhesive plate film (PlateMax: Axyseal Sealing Film, pre-sterilized PCR-SP) was placed over the wells, and sealed by hand. The plate was incubated (New Brunswick Scientific, model # G-25) for 24 hrs at 37°C with shaking at 200 rpm. After incubation 25 µL from each well was transferred into a matching sterile, 96 well filter plate (0.2 um PVDF membrane, Corning, product # 3504). To each well, 100 µL of TSB + 0.5%YE + 0.5% Tween 20 was added. A sterile 96 well plate lid was placed on the filter plate. The filter plate was then placed on the collection plate (Corning, 0.5 ml, product #3956) and centrifuged (Beckman TJ-6 centrifuge) at 2000 rpm for 10 min. After centrifugation, 100 µL of TSBYE + 0.5% Tween 20 was added to each well and then centrifuged again. After centrifugation, 200 µL of TSB + 0.5% YE + 1% TTC were added to each well. With a multichannel pipette, the contents of each well were gently mixed and transferred to matching sterile 96 well plate. The plate was statically incubated at 37°C for 48 hrs. The wells were then checked for growth via development of red pigment. Wells with the lowest oil concentration oil having no red pigment were considered MICs. The experiment was replicated four times.

Statistical Analysis

The data were compiled in Excel 2007. The repetitions were averaged and standard deviations were calculated for the means. Means were then examined using a Student's T Test to determine significant differences.

Results and Discussion

The results of the minimum inhibitory concentration determinations can be found in Table 1. Cold pressed terpeneless Valencia orange oil exhibited MICs of 0.5±0.0%, 0.5±0.0%, 0.31±0.13%, and 0.31±0.13% against *E. coli* O157:H7, *L. monocytogenes, S. aureus,* and *S.*Typhimurium, respectively. MICs of 0.75±0.29%, 0.31±0.13%, 0.63±0.25%, and 0.44±0.13% were exhibited against *S. sonnei*, *Y. enterocolitica*, *E. faecalis*, and *B. cereus*, respectively. *P. aeruginosa* was significantly more resistant to the oil ($p<0.05$) than the rest of the bacteria tested. It exhibited complete resistance to the oil, even at a concentration of 10%. The minimum inhibitory concentrations of *S. aureus, S.* Typhimurium*,* and *Y. enterocolitica* were found to be significantly lower than that of *S. sonnei* ($p<0.05$). The MICs determined for *E. coli* O157:H7, *L. monocytogenes*, *E. faecalis*, and *B. cereus* were not found to be significantly different from each other, or significantly different from *S. aureus*, *S.* Typhimurium*, S. sonnei*, or *Y. enterocolitica* ($p>0.05$).

Prior studies have evaluated the effects of cold pressed terpeneless Valencia orange oil against different bacteria using non-membrane filtration techniques (O'Bryan et al. 2008, Nannapaneni et al. 2008, and Friedly et al. 2009). Friedly et al. (2009) examined the effect of the essential oil on strains of *L. monocytogenes* and *Listeria innocua*. It was determined that the oil exhibited a MIC of 0.55% against both *L. monocytogenes* and *L. innocua* after 18 hours of incubation at 37°C. This result is very similar to the result found in the current study, indicating

that the antibacterial inhibition of *L. monocytogenes* by the oil is not influential after 18 hours of incubation at 37°C. O'Bryan et al. (2008) determined the antibacterial effect of the oil against strains of *Salmonella,* including a strain of *S.* Typhimurium (Copenhagen). Cold pressed terpeneless Valencia orange oil exhibited little effect on the strain of *S.* Typhimurium tested. The zone of inhibition determined by disc diffusion was only 7.3±1.2 mm, which included the 6 mm paper disc. The results of the current study do not agree with this lack of antibacterial activity against *S.* Typhimurium. This could possibly be due to differences between strains of Typhimurium, although further research is needed to confirm this hypothesis. Nannapaneni et al. (2008) examined the ability of cold pressed terpeneless Valencia orange oil to inhibit the growth of *E. coli* O157:H7 strains. The oil, tested by disc diffusion, produced a zone of inhibition against *E. coli* O157:H7 (ATCC 43888) of 11.5±0.7 mm. It is not stated in the study if the inhibition zone includes the paper disc, but even if it does, the oil exhibited a much higher inhibition of *E. coli* O157:H7 (ATCC 43888) than *S.* Typhimurium (Copenhagen). This result is contradicted by the current study, in which both *E. coli* O157:H7 (ATCC 43888) and *S.* Typhimurium were found to be inhibited at the same essential oil concentration. The contrast in results could be due to the difference in methods used to determine antibacterial susceptibility, as well as a difference in strains of *S.* Typhimurium. The current study used a micro-broth dilution method, while the other studies used disc diffusion methods. The activity of cold pressed terpeneless Valencia orange oil possibly could be increased in a broth medium, although further study is needed to elicit an answer.

The responses of the other bacteria used in this study to cold pressed terpeneless Valencia orange oil have not been previously determined. Therefore, it is not possible to compare the results of this study to others. It can be said that the current method was able to elicit MICs for all other bacteria tested, except *P. aeruginosa*. The inability to determine an MIC

for *P. aeruginosa* is most likely due to its inherent resistance to essential oil components (Cox and Markham 2007). Cox and Markham (2007), found that *P. aeruginosa* NCTC 9027 and 6749 were tolerant of 6 of 8 essential oil components. Only cinnamaldehyde and carvacrol showed any inhibition of the bacteria. It was determined that these bacteria contained an active efflux mechanism, which allows them to resist the antibacterial effects of many essential oil components.

Conclusion

The current method tested was able to produce reliable MICs, but was unable to verify the need for membrane filtration in the MIC determinations of essential oils. The MICs determined in this study were either similar to those found by traditional methods, or lower than those found by traditional methods. These results are contrary to that of the original hypothesis: MICs should be lower in traditional methods due to residual inhibition by essential oils. Replication of these results is needed to confirm this conclusion.

Acknowledgements

This research was funded by a National Cattleman's Beef Association grant to author Goodridge and USDA/NIFSI Grant # 2008-51110-04339 to authors Ricke and Crandall.

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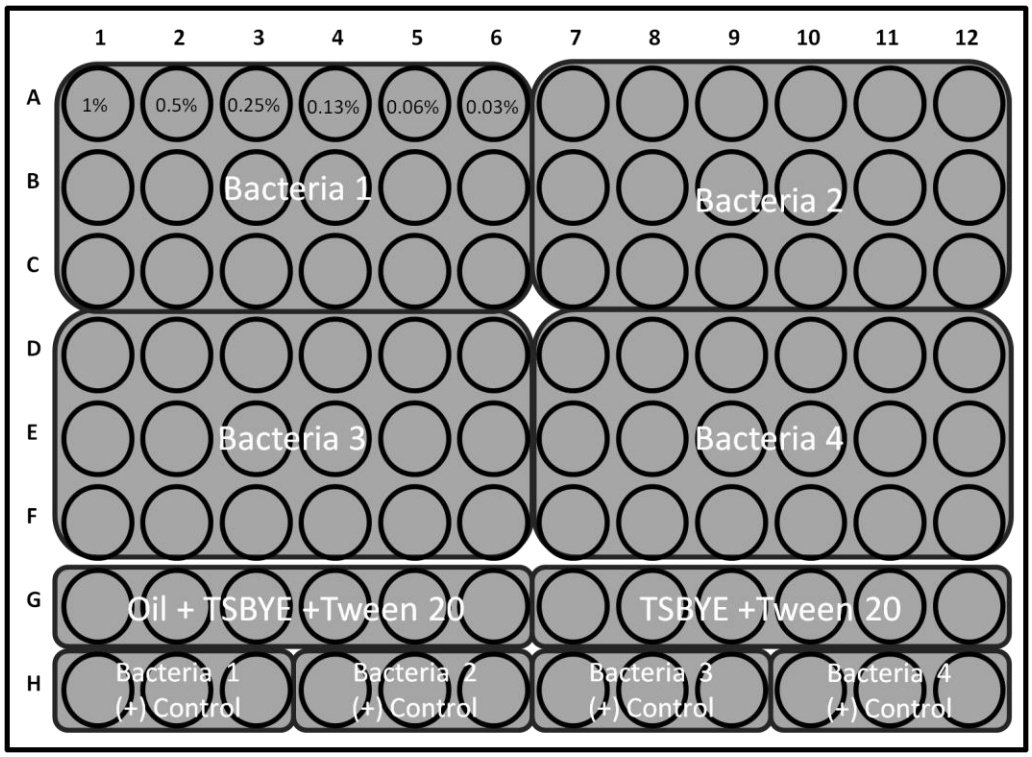

Figure 1. 96-well microtiter plate design for MICs with cold pressed terpeneless Valencia orange oil concentrations in percentages.

Table 1. Minimum inhibitory concentrations of cold pressed Valencia orange oil for 9 bacterial strains at 37°C.

Bacterial Strain	MIC (%)
E. coli O157:H7	0.5±0.0[1]
L. monocytogenes	0.5±0. 0
S. aureus	0.31±0.13
S. Typhimurium	0.31±0.13
S. sonnei	0.75±0.29
Y. enterocolitica	0.31±0.13
E. faecalis	0.63±0.25
B. cereus	0.44±0.13
P. aeruginosa	>10

[1]Mean±SD

Conclusions

In the beef industry, carcasses are chilled to temperatures close to or at 4°C within 24 hours. Our results indicated that a solution of 1% cold pressed terpeneless Valencia orange oil could be used as an additional intervention against *E. coli* O157:H7 at the chilling stage of processing, and will remain effective if the carcasses are subjected to temperature abuse. Further research into additional temperatures and strains are needed, as well as research into the actual application of cold pressed terpeneless Valencia orange oil onto beef carcasses during the chill stage. Further studies indicating the validity of this intervention point and antimicrobial will result in safer beef products, which will in turn lead to less recalls and more savings for those in the beef processing industry.

Membrane filtration allows one to remove cells from an antibacterial test solution, and it offers promise in producing more accurate results for MIC determinations of essential oils. While the membrane filtration method was able to produce reliable MICs, the results did not indicate the need for membrane filtration when determining MICs of essential oils. The MICs determined in the study were either similar to or lower than those found by broth dilution and disc diffusion methods in prior studies. Further research is needed to confirm these results.